J. Orchid Soc. India, 37: 77-80, 2023 ISSN 0971-5371

EXTRACELLULAR ENZYMATIC ACTIVITIES OF THE FUNGAL ASSOCIATES OF *DENDROBIUM OVATUM* (L.) KRAENZL., AN ENDEMIC ORCHID OF PENNINSULAR INDIA

Reedhu Raj, Prasanna Rajan[1], Sijo Mathew[1], Shanty Joseph[2], Joseph Job[3], and Elizabeth Cherian[2]

PG and Research Department of Botany, Govt. Victoria College, University of Calicut, Palakkad- 678 001, Kerala, India
[1]Department of Botany, Government College, Kottayam- 686 001, Kerala, India
[2]PG and Research Department of Botany, CMS College, Kottayam- 686 001, Kerala, India
[3]PG and Research Department of Botany, St. Berchmans College, Changanacherry- 686 101, Kerala, India

Abstract

The microbiome associated with any plant plays a significant role in the life cycle of its host by performing various plant-beneficial activities. They stimulate the growth and productivity of the host plant and defend the attack of phytopathogens. Moreover, they act as a reservoir of biologically and pharmacologically important enzymes, which may find many industrial applications. Orchids are at the front line of extinction, with more species under threat globally than any other plant family. Hence, they may be considered as the indicators of the health of any ecosystem as their existence depends on many other life forms, especially fungi, owing to their role in orchid seed germination. The present study focuses on isolating and identifying the fungi associated with the absorption roots of the wild epiphytic orchid, *Dendrobium ovatum*. Morphological characters and molecular characterisation using the ITS primers were used to confirm the identity of the isolates. *Trichoderma* sp. was found to be the dominant one. Capability of the isolates to produce extracellular enzymes like amylase, cellulase, lipase, protease, laccase, and tyrosinase were analysed. The results revealed the vast gamut of beneficial attributes possessed by the fungi associated with the absorption roots of *D. ovatum*. The present results highlighted the importance of conservation of *D. ovatum* while considering the diverse microbial associates and the microhabitat it dwells.

Introduction

ORCHIDS ARE one of the largest group of flowering plants with diverse kind of species found in all habitats except extremes of terrestrial environmental conditions (Bayman and Otero, 2006). *Dendrobium* is one of the largest genera of orchids, with 1,606 species (POWO, 2023). It is widely appreciated for its blooms and therapeutic qualities. Some reports have established the immunomodulatory, antidiabetic, hepatoprotective, anti-inflammatory, antioxidant, antimalarial, antibacterial, and antifungal activities of *Dendrobium* (Paul *et al*., 2022; Sanjana *et al*., 2021; Teixeira da Silva and Ng, 2017). *Dendrobium ovatum*, commonly known as the Green Lipped *Dendrobium* is the least studied species. It is an epiphytic orchid endemic to peninsular India and distributed widely in the Western Ghats, Eastern Ghats, and Deccan Plateau. Its state wise analysis showed that its distribution is restricted to the states of Kerala, Tamil Nadu, Karnataka, Andhra Pradesh, Goa, Gujarat, and Madhya Pradesh. It is used as a substitute for Jivanti in Ayurveda and its fresh juice serves as a liver tonic, laxative, stomachic, and carminative.

The associated microbiota has been considered to be a potent producer of extracellular enzymes for their inherent needs. Microbial derived enzymes are active and stable over a wide range of conditions when compared with plant and animal derived ones (Jalgaonwala and Mahajan, 2011). Moreover, they are preferred in multiple sectors in view of their stability, inexpensive substrates, high growth rate, and shorter generation times. Another feature is the very resilient nature of these enzymes to the fluctuations in pH, temperature, and salinity. Hence, identification and incorporation of these new enzymes with high stability for industrial processes is essential. The present study aims to examine the capability of fungal assemblages associated with *D. ovatum* in producing various extracellular enzymes.

Material and Methods

Fungal Associates

The fungal strains isolated from both the surface sterilised and non-surface sterilised segments of absorption roots of *D. ovatum*, collected from different parts of Pathanamthitta district, Kerala, were used in the present study. For the species-level identification of fungal isolates, the Internal Transcribed Spacer (ITS) region or large subunit (LSU) of the rRNA was sequenced. The consensus sequence so obtained was subjected to homology searches using Basic Local Alignment Search Tool search (www.ncbi.nlm.nih.gov/Blast) against the sequences in the database and the closest relative in the database was identified.

Received: April 5, 2023; Accepted: August 6, 2023

Extracellular Enzymatic Activities of the Isolates

Fungal cultures were grown in Potato Dextrose Agar (PDA) medium for seven days, after which 5 mm fungal plugs were placed on specific indicative media as proposed by Hankin and Anagnostakis (1975) and Uzma *et al*. (2016): starch to test amylolytic activity, carboxymethyl cellulose (CMC) to test cellulolytic activity, gelatin to test proteolytic activity, 1- napthol to test laccase activity, peptone agar medium for lipolytic activity, potassium iodide-iodine solution to test pectinase activity, and p-cresol to test tyrosinase activity. After 5 to 7 days of incubation, zone of clearance surrounding the fungal colony was noted (Sunitha *et al*., 2013).

Statistical Analysis

All the tests were performed in triplicates and the statistical analysis was performed using R program version 4.2.1 [R Core Team (2022)]. To test the normal distribution of data, Shapiro-Wilk normality test was performed. The comparisons were made by one way ANOVA followed by Tukey's HSD test with significance set at 0.05.

Results and Discussion

Dendrobium is a genus that is highly prized for its varied flowers and medicinal properties. The present study unveiled the capacity of fungal strains isolated from the absorption roots of *D. ovatum* to exhibit proficiency in the production of extracellular enzymes. Comparing the sequences of the Internal Transcribed Spacer (ITS) region or large subunit (LSU) of the rRNA against the database of BLAST, *Trichoderma* was identified as the most common isolate. *Collectotrichum* sp. and *Fusarium oxysporum* was isolated as endophytes of *D. ovatum* roots along with *T. viride* and *T. asperellum*.

During the present study, the isolation and identification of nine fungal strains was done using a combination of morphological characters and molecular characterisation using ITS primers from the absorption roots of *D. ovatum*. Majority of the members belonged to the genus *Collectotrichum* sp., *Fusarium* sp., and *Trichoderma* sp. (Table 1). The qualitative assessment of fungal isolates in producing extracellular enzymes was carried out by agar plate method. The ability of fungal strains, particularly *Trichoderma* to produce extracellular enzymes such as cellulases, proteases, and amylases have been known for a long time. The current study revealed that each isolate has the ability to generate at least one of the extracellular enzymes, but it was noted that none of the isolates exhibited the capacity to produce all the extracellular enzymes, concurrently. Amongst the tested isolates, 77% of the isolates were found to be producing amylase and lipase, 55% were cellulase producers, 44% produced pectinase, and only 11% produced protease and tyrosinase (Table 1). None of the isolates produced laccase, that degraded lignocellulosic materials. The present findings are consistent with the findings of Uzma *et al*. (2016), who attributed the lack of laccase activity to their endophytic origin of fungi as an active enzyme may damage the host itself.

The surface associated *Trichoderma* strains were eminent producers of amylase and cellulase when compared with the endophytic ones. It is noteworthy that the endophytic and surface associated *T. asperellum* displayed a remarkable difference in the production of amylase enzyme. While the surface associated *T. asperellum* displayed no amylase production, the endophytic *T. asperellum* showed a yellow zone around the colony. This may be aiding the endophytes in nutrient acquisition and thriving in nutrient deficient conditions of growth.

Surface associated *T. reesei* was found to be the prominent producer of cellulase along with *T. lentiforme*, which is in accordance with the fact that it is capable of producing excess amount of extracellular cellulolytic enzymes as compared to other *Trichoderma* strains. This character has led to the production of mutant *T. reesei* strains that are employed in various industrial and biotechnology fields for the production of cellulolytic and hemicellulolytic enzymes (Strakowska *et al*., 2014).

Protease was produced by the surface associated *T. lentiforme* only. None of the endophytic strains produced protease. Both the surface associates as well as the endophytic strains produced lipase, indicative of their ability to utilise fats as energy source. Tuangporn *et al*. (2012) reported *Fusarium oxysporum* as an eminent producer of laccase amongst the endophytes isolated from *Croton oblongifolius*. The present study indicated that *F. oxysporum* is an eminent producer of laccase after *T. lixii*. Only surface associated *T. lixii* produced tyrosinase enzyme. These seem to aid the fungi in nutrient acquisition and defence against competitors.

The production of extracellular enzymes by the associated microbiota aids them in performing a plethora of plant beneficial activities like disease suppression, induction of disease resistance, hyper parasitic activity *etc*. In addition, they also help the microbiota in cleavage of plant cell wall, thus facilitating their entry into the host (Khalil *et al*., 2021). According to Tchameni *et al*. (2020), the production of cellulases, proteases, chitinases, and

Table 1. Extracellular enzymatic activity of the fungal associates procured from the absorption roots of *D. ovatum*.

Nature	Isolate name	Fungal isolate	Family	Diameter of clear zones (mm)					
				Amylase	Cellulase	Protease	Lipase	Tyrosinase	Pectinase
Surface Associate	DNST 01	*Trichoderma harzianum*	Hypocreaceae	0^{g}	0^{c}	0^{b}	39.67 ± 1.15^{b}	0^{b}	71.67 ± 1.53^{a}
	DNST 02	*T. lentiforme*	Hypocreaceae	24.0 ± 1.0^{f}	35.0 ± 2.0^{a}	32.66 ± 0.58^{a}	46.33 ± 2.08^{a}	0^{b}	0^{d}
	DNST 03	*T. reesei*	Hypocreaceae	53.67 ± 1.15^{b}	35.0 ± 2.0^{a}	0^{b}	21.67 ± 1.53^{c}	0^{b}	0^{d}
	DNST 05	*T. asperellum*	Hypocreaceae	0^{g}	0^{c}	0^{b}	23.33 ± 1.53^{c}	0^{b}	0^{d}
	DNST 06	*T. lixii*	Hypocreaceae	31.33 ± 0.58^{d}	31.66 ± 2.08^{a}	0^{b}	43.67 ± 1.53^{a}	53.33 ± 1.15^{a}	17.66 ± 0.58^{c}
Endophyte	DST01	*Collectotrichum* sp.	Glomerellaceae	37.33 ± 1.15^{c}	0^{c}	0^{b}	0^{d}	0^{b}	71.33 ± 1.53^{a}
	DST02	*Fusarium oxysporum*	Nectriaceae	29.0 ± 1.00^{e}	19.33 ± 1.53^{b}	0^{b}	38.33 ± 0.58^{b}	0^{b}	72.33 ± 0.58^{a}
	DST04	*T. viride*	Hypocreaceae	28.67 ± 0.58^{e}	0^{c}	0^{b}	36.67 ± 1.15^{b}	0^{b}	0^{d}
	DST05	*T. asperellum*	Hypocreaceae	59.67 ± 0.58^{a}	18.66 ± 1.53^{b}	0^{b}	0^{d}	0^{b}	53.33 ± 0.58^{b}

All the values are means of triplicates±S.D., Mean values in the column that does not share a common lowercase superscript differ significantly at $P<0.05$, according to Tukey's HSD test.

xylanases by *Trichoderma* is responsible for the antagonistic activity against pathogens.

Conclusion

Microbial enzymes have now become an attractive alternative to plant or animal enzymes due to their stability, eco-friendly production, and tailored properties to meet specific industrial needs. Fungal enzymes are more preferred over the bacterial enzymes owing to their consideration as GRAS (generally recognised as safe) and easy separation from the fermentation broth (Bezerra *et al*., 2021). The variation in the capability of isolates to produce extracellular enzymes may be attributed to the host specific and ecological niche specific factors. Thus, the search for new sources of these enzymes can have tremendous impact in various industrial segments.

Acknowledgments

All the authors acknowledge the head of the institutions of respective centres and the Director of Collegiate Education, Kerala, India.

References

Bayman, P. and J. T. Otero. 2006. Microbial endophytes of orchid roots. *In*: *Microbial Root Endophytes*, pp. 153-77. Springer, Heidelberg, Berlin.

Bezerra, V. H. S., S. L. Cardoso, Y. Fonseca-Bazzo, D. Silveira, P. O. Magalhães, and P. M. Souza. 2021. Protease produced by endophytic fungi: A systematic review. *Molecules,* **26**(22): 7062. https://doi.org/10.3390/molecules26227062.

Hankin, L. and S. L. Anagnostakis. 1975. The use of solid media for detection of enzyme production by fungi. *Mycologia,* **67**: 597-607. https://doi.org/10.1080/00275514.1975.12019782.

Jalgaonwala, R. E. and R. T. Mahajan. 2011. Evaluation of hydrolytic enzyme activities of endophytes from some indigenous medicinal plants. *J. Agric. Technol.*, **7**: 1733-41.

Khalil, A. M. A., S. E. D. Hassan, S. M. Alsharif, A. M. Eid, E. E. D. Ewais, E. Azab, A. A. Gobouri, A. Elkelish, and A. Fouda. 2021. Isolation and characterization of fungal endophytes isolated from medicinal plant *Ephedra pachyclada* as plant growth-promoting. *Biomolecules,* **11**(2): 140. https://doi.org/10.3390/biom11020140.

Paul, T., B. Chowdhury, T. K. Bhowmik, and M. M. Rahman. 2022. Antioxidant and anti-inflammatory activities of *in vivo* grown plants and *in vitro* raised plantlets in *Dendrobium crepidatum* Lindl. & Paxt. of Bangladesh. *J. Orchid Soc. India*, **36**: 29-33.

POWO. 2023. *Plants of the World Online.* Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://www.plantsoftheworldonline.org/.

Sanjana, Tahli, M. M. Hoque, and M. K. Huda. 2021. Antioxidant and anti-inflammatory potential of an epiphytic and endangered orchid, *Dendrobium moschatum* (Buch.-Ham.) Sw. *J. Orchid Soc. India*, **35**: 19-23.

Strakowska, J., L. Blaszczyk, and J. Chelkowski. 2014. The significance of cellulolytic enzymes produced by *Trichoderma* in opportunistic lifestyle of this fungus. *J. Basic Microbiol.,* **54**: 1-12. https://doi.org/10.1002/jobm.201300821.

Sunitha, V. H., D. N. Devi, and C. Srinivas. 2013. Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. *World J. Agr. Sci.*, **9**: 1-9. https://doi.org/10.5829/idosi.wjas.2013.9.1.72148.

Tchameni, S. N., M. Cotârle, I. O. Ghinea, M. A. B. Bedine, M. L. Sameza, D. Borda, G. Bahrim, and R. M. Dinica. 2020. Involvement of lytic enzymes and secondary metabolites produced by *Trichoderma* spp. in the biological control of *Pythium myriotylum*. *Int. Microbiol.*, **23**: 179-88.

Teixeira da Silva, J. A. and T. B. Ng. 2017. The medicinal and pharmaceutical importance of *Dendrobium* species. *Appl. Microbiol. Biotechnol.,* **101**: 2227-39. https://doi.org/10.1007/s00253-017-8169-9.

Tuangporn, Panuthai, P. Sihanonth, J. Piapukiew, S. Sooksai, P. Sangvanich, and A. Karnchanatat. 2012. An extracellular lipase from the endophytic fungi *Fusarium oxysporum* isolated from the Thai medicinal plant, *Croton oblongifolius* Roxb. *Afr. J. Microbiol. Res.*, **6**: 2622-38. https://doi.org/10.5897/ajmr11.965.

Uzma, F., N. M. Konappa, and S. Chowdappa. 2016. Diversity and extracellular enzyme activities of fungal endophytes isolated from medicinal plants of Western Ghats, Karnataka. *Egypt. J. Basic Appl. Sci.*, **3**: 335-42. https://doi.org/10.1016/j.ejbas.2016.08.007.